Membrane proteomics of phagosomes suggests a connection to autophagy

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Phagocytosis is the central process by which macrophage cells internalize and eliminate infectious microbes as well as apoptotic cells. During maturation, phagosomes containing engulfed particles fuse with various endosomal compartments through the action of regulatory molecules on the phagosomal membrane. In this study, we performed a proteomic analysis of the membrane fraction from latex bead-containing (LBC) phagosomes isolated from macrophages. The profile, which comprised 546 proteins, suggests diverse functions of the phagosome and potential connections to secretory processes, toll-like receptor signaling, and autophagy. Many identified proteins were not previously known to reside in the phagosome. We characterized several proteins in LBC phagosomes that change in abundance on induction of autophagy, a process that has been previously implicated in the host defense against microbial pathogens. These observations suggest crosstalk between autophagy and phagocytosis that may be relevant to the innate immune response of macrophages.

LC3 | phagocytosis

Phagosomes are specialized membrane-bound organelles generated in phagocytic cells such as macrophages, neutrophils, and dendritic cells. Their purpose is to internalize foreign particles, microorganisms, or apoptotic cells, to mount an immune response, or to maintain tissue homeostasis (1). The nascent phagosome undergoes a complex maturation process involving sequential fusion with endosomal compartments. Once mature, the phagosome degrades its constituents and facilitates antigen presentation in a highly controlled manner (2–4). Insight into the biogenesis and immunity-related functions of the phagosome has come from analysis of its protein contents. Previous studies have profiled the proteomes of latex bead-containing (LBC) phagosomes in cell lines from mice (5–7) and *Drosophila sp.* (8), as well as *Dictyostelium discoideum* (9) and *Entamoeba histolytica* (10). These elegant studies contribute to our understanding of phagosome maturation (6, 9) and modulation by cytokines (7); however, because the entire contents of the phagosomes were sampled, abundant proteins, such as soluble lysosomal hydrolases, might have obscured lower abundance membrane-bound regulatory proteins or signaling factors.

To identify such lower abundance species, we enriched integral and peripheral membrane proteins from macrophage LBC phagosomes by organelle sub-fractionation, before carrying out a large-scale proteomic analysis. The 546 proteins identified in our study included 49 membrane receptors, 64 transporter proteins, 107 regulators of vesicle and protein trafficking (including GTPases), and many components from cellular machineries other than phagosomes. One of the new proteins exclusively found in our study, LC3-II, is considered a marker of autophagic activity. Its presence in phagosomes suggests an unexplored linkage between autophagy and phagocytosis. Apart from its housekeeping role in maintaining cellular homeostasis, autophagy has been implicated in cancer, neurodegerative disorders, aging, and, more recently, immunity against intracellular microbes (11–13). We show here that LC3-II levels in phagosomes are modulated by autophagic activity, along with several other proteins not previously associated with autophagy. These results underscore the power of membrane-specific phagosomal proteomics for identifying new processes in which this organelle may engage.

Results and Discussion

Phagosome Isolation and Membrane Fractionation. Fig. 1 illustrates our procedure for integrating sub-cellular fractionation techniques with the proteomic platform. We applied the method of Desjardins and coworkers (14) for phagosome isolation. Briefly, latex beads were internalized into macrophages and the latex bead-containing (LBC) phagosomes were isolated by flotation on a sucrose gradient. Phagosomes isolated in this manner are devoid of major contaminants (14, 15) and retain critical functional capabilities, such as sequential fusion with endocytic vesicles (16) and microbicidal activity (17). By using radiolabeling and proteomic analysis, Desjardins *et al.* (14) estimate the potential contamination of LBC phagosomes to be below 5%. The first proteomic study of LBC phagosomes, by using this method, identified ≈140 proteins. Not surprisingly, many of the observed proteins were highly abundant lysosomal hydrolases from the lumen of the vesicle.

To favor the recovery of integral or peripheral membrane proteins, which are at relatively low abundance, we lysed the phagosome pellet in sodium carbonate to release luminal proteins. The most loosely bound membrane-associated components were then depleted by a second centrifugation. It should be noted that several soluble proteins known to be transiently associated with phagosomal membranes participate in vesicle traffic and signaling (such as the Rab family) (18–20). In an effort to retain some of these functionally significant proteins, we refrained from extensive subsequent washing of the membrane fraction. The resulting insoluble pellet was re-suspended in concentrated detergent (4% SDS) to solubilize the residual proteins before separation by SDS/PAGE and identification by LC-MS/MS. Enrichment of unique proteins in the purified phagosomal membrane fraction, compared to other fractions, was confirmed by SDS/PAGE [supporting information (SI) Fig. S1].

To verify the purity of isolated phagosomes and their membrane fractions, we probed for the presence of known cellular organelle markers. GM-130 (Golgi-resident), Calnexin (ER-resident), and HSP-60 (mitochondria-resident) were detected

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The authors declare no conflict of interest.

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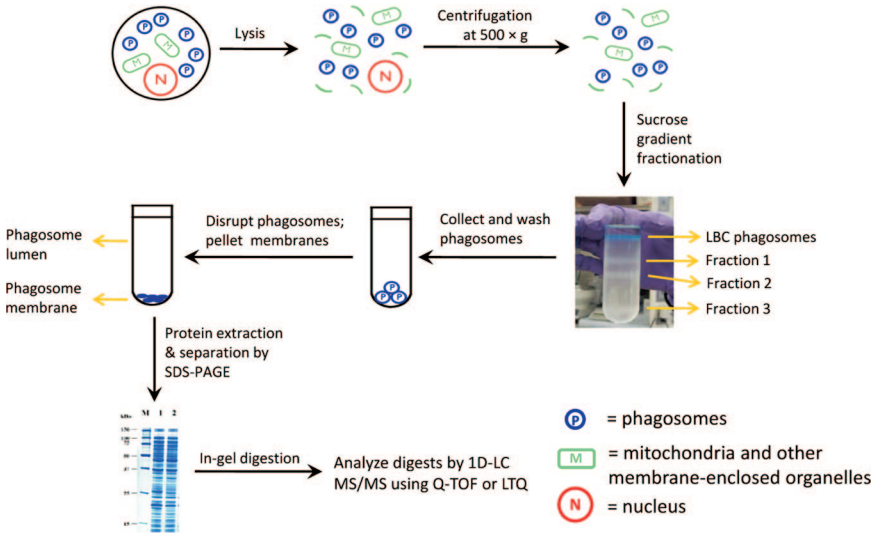

Fig. 1. Macrophage subcellular fractionation of phagosomal membranes, and proteomic analyses.

only in nonphagosomal fractions, whereas the lysosomal-associated membrane protein-1 (LAMP1) was clearly present in the phagosomal fraction, as expected from the process of phagosome–lysosome fusion (Fig. 2*A*). In the absence of latex beads, LAMP1 was not observed in the corresponding fraction that was obtained by centrifugation, thus confirming its association with phagosomal membranes.

We next evaluated the composition of the phagosomal membrane preparation with respect to membrane-associated versus soluble proteins. Western blot analysis demonstrated that three known membrane markers of endosomal compartments [early endosomal associated protein (EEA1), LAMP1, and transferrin receptor (TfR)] were more abundantly represented in the membrane extract than in the soluble luminal fraction (Fig. 2*B*). All 3 endosomal markers were also observed in other subcellular fractions that include endogenous endocytic vesicles. In contrast to membrane markers, a significant portion of the soluble phagosomal protease cathepsin D (CatD) was released into the lumen fraction. However, this luminal protein was also observed as a contaminant in our membrane fraction. Thus, although our method enriches membrane-bound proteins considerably, the membrane fraction is not free of soluble contaminants.

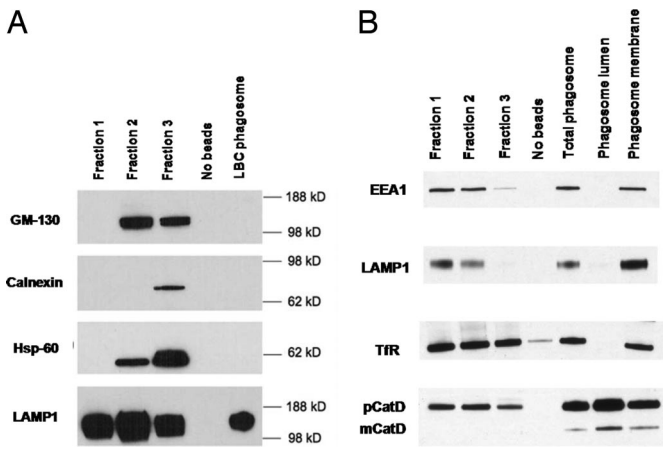

Fig. 2. Immunoblot analysis of subcellular fractions. (*A*) Blot probed for organelle markers. (*B*) Blot probed for membrane-bound endosomal markers and a lysosomal hydrolase. Fractions are denoted in Fig. 1. ''No beads'' indicates a sample taken from the same position occupied by LBC phagosomes in the sucrose gradient, yet derived from macrophages without beads. Each lane was loaded with 3 μg of total protein and equal loading was verified by silver stain (see Fig. S1).

Identification of Phagosomal Membrane-bound Proteins and Functional Categorization. Two types of mass spectrometers (Q-TOF and linear ion trap) were used to identify phagosomal membrane-bound proteins prepared in biological duplicates. The raw MS/MS data acquired from the two instruments were searched by a single engine (Mascot), and a stringent set of filter criteria was applied to select high-confidence protein IDs. A total of 546 nonredundant IDs were generated in the 2 experiments by using different instruments. The details of protein identification are listed in Dataset S1. Interestingly, when we compared our dataset with that from the recent study conducted by Foster *et al*. (6) that identified 505 proteins from entire phagosomes of a different mouse macrophage cell line (without membrane fractionation), 318 IDs were exclusively found in our dataset, and 277 IDs were unique hits revealed by Foster's experiment (6) (Fig. 3*A*). All of the 546 proteins identified in our analysis were categorized into 14 major classes according to specific cellular processes or functions annotated in public databases (Fig. S2 and Dataset S2). We also listed these proteins in order of relative abundance, as indicated by their ID score and number of identified peptides (Dataset S3).

Dataset S4 highlights unique proteins identified in the previously reported phagosomal proteome dataset, as well as the one we acquired here, with a summary of their molecular weights (MW) and number of predicted transmembrane helices (TMHs) based on primary sequences (Figs. 3 *B* and *C*, respectively). The histogram of MWs shows an overall similar distribution profile, although our dataset contains slightly more IDs >40 kDa than the previously reported phagosomal proteomic dataset (6). We found 198 hits with >1 predicted TMH, which constitutes 36% of the entire phagosomal proteome in our analysis. By comparison, 27% of the phagosomal proteome reported by Foster *et al*. (6) was predicted to comprise transmembrane proteins, and a proteomic profile of *Drosophila sp*. phagosomes estimated that value at 19.8% (8). Furthermore, proteins with more than 1 TMH were represented at higher frequency in our unique dataset compared to unique proteins in the previously reported datasets. Among our uniquely identified proteins are ion channel and solute carrier proteins, a class of predicted transmembrane proteins with unknown function, as well as members of the

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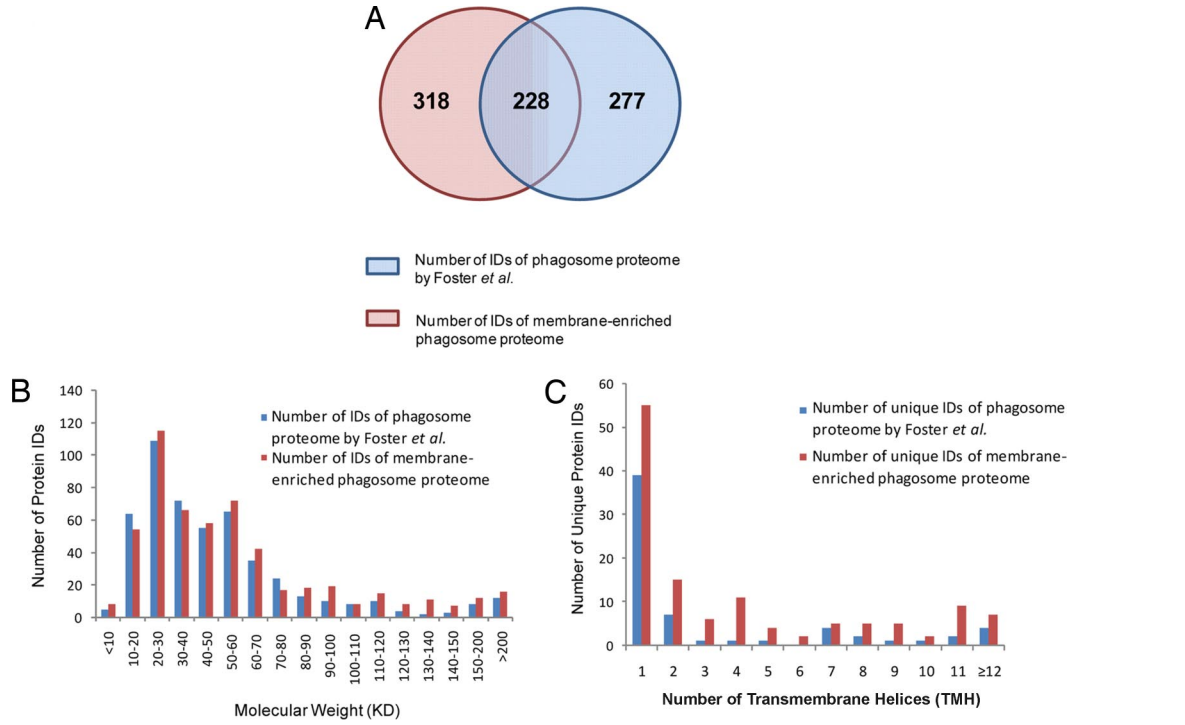

Fig. 3. Comparison of this membrane-enriched phagosomal proteomic data to the reported total phagosomal proteomic data. (*A*) Venn diagram of total protein IDs from this membrane proteomic dataset and Foster's comprehensive proteomic dataset for LBC phagosomes (6). (*B*) Histogram of predicted molecular weights of the proteins in the 2 datasets. (*C*) Histogram of the number of predicted TMH in the unique proteins that were identified in the 2 datasets. Unique IDs refers to proteins exclusively reported in 1 dataset.

vesicle-associated membrane protein (VAMP) family (Dataset S4). Overall, comparison of the identity and physical properties of proteins from the 2 datasets indicates that: (a) membrane enrichment allows for discovery of more transmembrane proteins without significantly altering MW distribution, and (b) two separate experiments with different cell lines, by using different mass spectrometers, generated many unique IDs.

Validation of Newly Identified Phagosomal Components. We selected several proteins for further validation by immunoblotting. Some were previously revealed in the earlier study by Desjardin (5), thus providing a measure of further validation, whereas others were newly identified in this study. As shown in Fig. 4, VAMP4, toll-like receptors (TLR)7 and TLR9, Janus kinase 1 (JAK1), and microtubule-associated protein 1/light chain 3 (LC3) were more enriched in the phagosomal membrane fraction compared to other subcellular fractions or total cell lysate. Foster *et al*. (6) also identified VAMP4 in macrophage phagosomes by mass spectrometry, and certain TLRs were similarly identified by Desjardin (7) in a recent study of macrophage activation by cytokines. By contrast, JAK1 and LC3, which participate in immune signaling and autophagy, respectively, had not been identified previously in phagosomes. The functional implications of each of these findings are summarized below.

VAMP4 is a member of the soluble *N*-ethylmaleimide-sensitive attachment receptor (SNARE) protein family that mediates intracellular membrane trafficking and fusion (21). VAMP4 is associated with the trans-Golgi network (TGN) and immature secretory granules (22) and is known to mediate trafficking of the sorting and recycling endosomes (23). The protein has not been directly implicated in phagocytosis, but enrichment of VAMP4 on LBC phagosomes suggests a potential role in this pathway. In addition to VAMP4, many other annotated proteins with relevance to "vesicle and protein trafficking" (Dataset S2), such as VAMP3, t-SNARE-interacting proteins, and Sec family members, were identified in our study. Their potential contribution to the biogenesis or function of macrophage phagosomes is an interesting future avenue of investigation.

The TLR family plays a critical role in innate immunity by recognizing a diverse range of microbial components (24). Several TLR members were identified in our study, as summarized in Table 1. TLR3, 7, and 9 are intracellular receptors that sense bacterial or viral nucleic acids in endosomal compartments, but their relation to phagocytosis is not well understood.

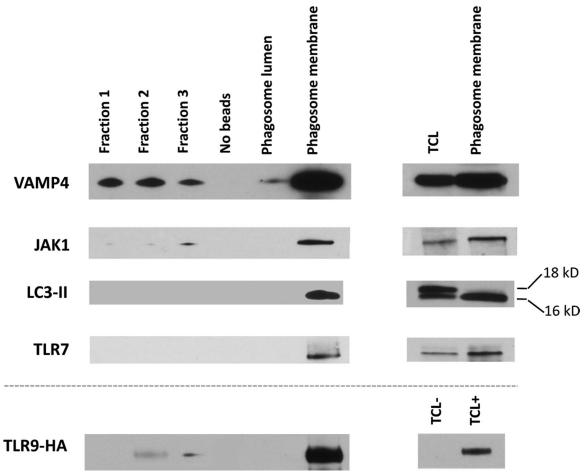

Fig. 4. Immunoblots of proteins in phagosomal membranes. TCL+ = total cell lysate obtained from RAW cells expressing HA-TLR9; TCL− = total cell lysate from normal RAW cells. Anti-HA was used to probe for TLR9. Three μg of each sub-cellular fraction was loaded for detection of VAMP4, LC3, and JAK1, whereas 7 μg was loaded for TLR7 and TLR9 detection. All of the total cell lysates were loaded at 15 μg.

Table 1. Identification of TLR family members in the membrane proteome of LBC phagosomes

Protein name	IPI accession no.	Major ligands in innate immunity	Predicted protein MW, KD	Mascot score of protein ID	No. of peptide IDs	Relative abundance index*
TLR 2	IPI00131898	Lipids and glycans from bacteria and fungi	91	152	6	3.6
TLR 3	IPI00320618	dsRNA from viruses	104	108	4	3.8
TLR 7	IPI00122181	ssRNA from viruses	123	897	47	38
TLR 9	IPI00318748	DNA from viruses and bacteria	118	146	10	8.5
TLR 13	IPI00342691	Unknown	115	1160	52	45

*Calculated by dividing "No. of peptide IDs" with "Predicted protein MW", then multiplying by 100.

By using proteomic techniques, Desjardin (7) found these three TLRs to be up-regulated within macrophage phagosomes upon IFN-γ treatment. Surprisingly, an uncharacterized TLR member, TLR13, was identified in our study and its relative abundance, estimated by normalizing the number of identified peptides with the theoretical protein size (25), was significantly higher than all of the other TLRs found in phagosomes (Table 1). This observation suggests that further exploration of the role of TRL13 in phagocytosis is warranted.

JAK1, a protein tyrosine kinase, is known to associate with the cytoplasmic tails of cytokine receptors and plays a crucial role in initiating JAK/STAT signaling for enhancing microbial killing, antigen presentation, and inflammatory cytokine production (26). Fluorescence microscopy analysis of a JAK1-YFP fusion protein showed it to be localized primarily at the plasma membrane (27). However, we identified native JAK1 in the phagosomal membranes of macrophage cells, suggesting a possible role in either immune defense or other functions of this organelle.

Finally, LC3 piqued our interest because it is a widely used marker and an essential component of the autophagic machinery. On the induction of autophagy, the 18-kDa cytosolic precursor, LC3-I, is cleaved at its C-terminus and conjugated to phosphotidylethanolamine, generating a 16-kDa form termed LC3-II (28). Lipid-modified LC3-II integrates into the membrane of autophagosomes and undergoes either recycling or degradation when autophagosomes fuse with late endosomes (13). Interestingly, we observed the specific enrichment of LC3-II on LBC phagosomal membranes, whereas both forms of LC3 were observed in the total cell lysate (Fig. 4). It is unlikely that LC3-II found in LBC phagosomes came from contamination of the preparation with autophagosomal membranes. LBC phagosomes are known to be devoid of double-membrane vacuoles (a key feature of autophagosomes) and other major cellular compartments (5, 8, 14, 15). The finding of LC3-II in LBC macrophages prompted us to explore the link between phagosomal components and autophagy more closely.

Regulation of Phagosomal Components upon Autophagy Induction. Autophagy is a fundamental homeostatic process that enables cells to (a) either clean up or turn over portions of their own cytoplasm, mainly to obtain nutrients, (b) remove damaged organelles, or (c) remove toxic macromolecules (29). Autophagic functions are broadly associated with control of cell development, suppression of tumorogenesis, prevention of neurodegeneration, and immune response (11–13). On the initiation of autophagy, discrete portions of the cytoplasm are sequestered into a membrane-enclosed vacuole termed the autophagosome. The cytosolic components, or organelles trapped within a nascent autophagosome, are eventually degraded after their fusion with late endosomes (13).

To assess the relationship of phagosomal LC3-II and autophagic activity, we induced autophagy by nutrient starvation (30), in the presence and absence of pharmacological modulators, and monitored LC3-II levels within LBC phagosomes by western blot analysis. Vinblastine was used to accumulate autophagosomes, by preventing their fusion with endosomal compartments, and protease inhibitors were used to accumulate autophagosomal components, by preventing their degradation within autolysosomes (31). Furthermore, we used 3-methyladenine (3-MA), which blocks class III PI3Ks, as an inhibitor of the autophagic pathway (32, 33). The effectiveness of our phagosomic purification from these autophagy-regulated cells was verified by the observation of similar patterns of organelle marker distribution in subcellular fractions between control and drug-treated cells (Fig. S3).

As expected, LC3-II from total cell lysates of starved macrophages was more abundant in the presence of vinblastine or protease inhibitors than in their absence (Fig. 5*A*), consistent with the accumulation of autophagosomes. We also found LC3-II levels in LBC phagosomes to be elevated in the presence of vinblastine and protease inhibitors. Because LC3-II is predominantly associated with autophagosomes in nonphagocytic cells, and vinblastine blocks fusion of autophagosomes with

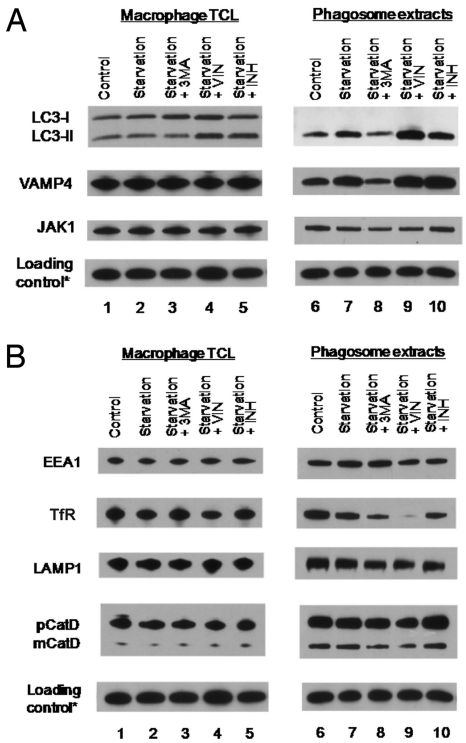

Fig. 5. Regulation of phagosomal proteins in response to autophagy induction. Immunoblots of (*A*) phagosomal components and (*B*) endosomal markers, in either macrophage total cell lysate (TCL) (*Left*) or phagosomal extracts (*Right*). Macrophages were subjected to treatment with rich medium (lanes 1 and 6), starvation (lanes 2 and 7), starvation and 10 mM 3-methyladenine (3-MA) (lanes 3 and 8), starvation and 50 μM vinblastine (VIN) (lanes 4 and 9), and starvation and protease inhibitors (INH) (lanes 5 and 10). The loading control was tubulin for macrophage TCL and actin for phagosome extracts.

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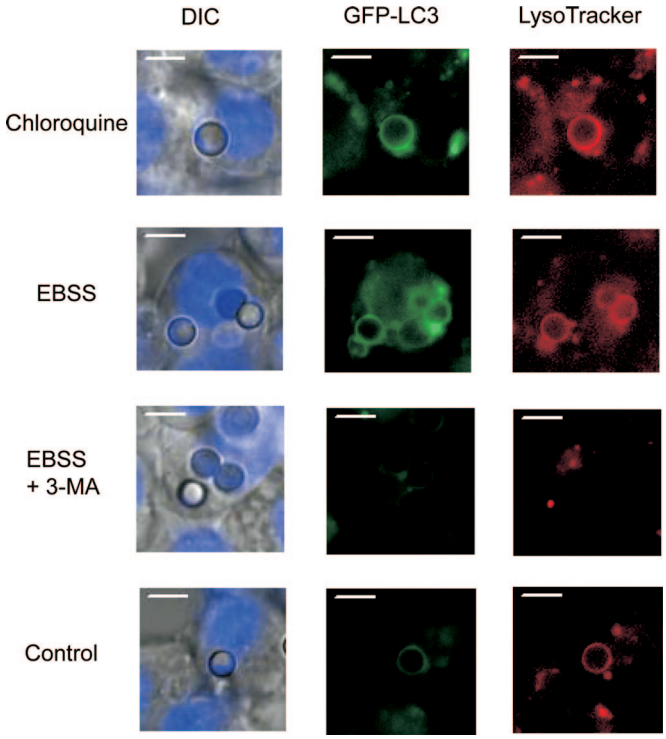

Fig. 6. Translocation of GFP-LC3 to LBC phagosomes during autophagy induction. RAW cells that stably express GFP-LC3 were allowed to internalize latex beads (3 μm) for 1 h, and then incubated with medium containing the lysosomal protease inhibitor, chloroquine (50 μM), EBSS buffer alone, and EBSS containing 3-MA (10 mM), for 2 h. Control was treated with rich medium after bead internalization. LysoTracker was added in the last 15 min to stain acidic vacuoles. All cells were treated while alive with nuclear stain Hoechst 33342 (blue). Representative images of different samples are shown. (Scale bar, 5 μm).

endocytic compartments (28, 34), we suspect that LC3-II was directly transferred from autophagosomes to LBC phagosomes. Consistent with this hypothesis, 3-MA treatment, which inhibited autophagosome formation, reduced LC3-II levels within LBC phagosomes (Fig. 5*A*).

Fluorescence microscopy of RAW cells stably expressing GFP-LC3 was used to confirm the enrichment of LC3 on phagosomic membranes during autophagy induction. We observed a distinct translocation of GFP-LC3 to the outermost ring of latex beads, engulfed by macrophages treated with either chloroquine-containing medium or starvation buffer (Fig. 6). These LC3-enriched phagosomes underwent successful fusion with lysosomes to become acidic vacuoles, as indicated by LysoTracker stain (Fig. 6). In contrast, 3-MA treatment abolished both GFP-LC3 recruitment to phagosmes and LysoTracker staining because of inhibition of PI3K, which is an essential factor for autophagy induction, as well as H^+-ATPase complex assembly (35). Notably, we also observed weak LC3 staining around a portion of LBC phagosomes in control cells (Fig. 6), suggesting a low level of endogenous autophagic activity. This observation was consistent with our previous identification of LC3 in phagosome membranes from unstimulated macrophages by using the proteomic approach.

Similarly to LC3-II's response to autophagic activity, the amount of VAMP4 in LBC phagosomes increased drastically when autophagosomes were accumulated, and it decreased during inhibition of autophagy (Fig. 5*A*, lanes 6–10). This trend suggests trafficking of VAMP4 between the 2 types of phagocytic compartments. We attributed the change of VAMP4 levels in phagosomes to intracellular translocation because the total cellular level of this protein was observed to be constant (Fig. 5*A*, lanes 1–5). The specific role of VAMP4 in either phagocytosis or autophagy has not been elucidated and would be an intriguing subject to pursue. JAK1, another newly identified phagosomal component, did not show a dramatic change in either total expression or subcellular distribution upon autophagy induction (Fig. 5*A*).

We further probed the response of other identified phagosomal components, endosomal marker (EEA1), transferrin receptor (TfR), LAMP1, and lysosomal hydrolase cathepsin D (CatD), to autophagy induction. None of these proteins were found to be up-regulated in phagosomes upon autophagy activation, nor were their overall cellular expression levels significantly altered (Fig. 5*B*). These results suggest that LC3-II and VAMP4 are specifically regulated in response to autophagy. We also noticed that the relative levels of the endosomal and lysosomal markers within phagosomes differed from one another, particularly upon treatment of macrophages with vinblastine (Fig. 5*B*, lane 9). EEA1 was mostly retained in phagosomes, whereas TfR was nearly depleted during autophagosome accumulation induced by the drug. In contrast, CatD and LAMP1 were slightly down-regulated under the same treatment. The differential effects of vinblastine on recruitment of the various endosomal and lysosomal proteins to phagosomes might reflect distinct trafficking routes of the proteins between autophagosomes and LBC phagosomes.

Conclusions

Proteomic analysis of a purified membrane fraction from LBC phagosomes resulted in the identification of many new proteins, as well as clues regarding a relationship between phagocytosis and autophagy. The 2 types of phagocytic compartments involved in these processes, generated by seemingly different pathways, may undergo a direct fusion that allows them to exchange and degrade constituents. The molecular machinery underlying the putative fusion event would be of significant future interest. Autophagy is thought to facilitate the control and depletion of intracellular pathogens (36, 37). In this regard, the process has a functional relationship with phagocytosis, whereby microbes from the external environment are internalized and digested. A potential synergy between autophagocytosis and conventional phagocytosis of pathogenic microbes warrants further investigation.

Materials and Methods

Phagosome Isolation and Membrane Fractionation. The murine macrophage cell line, J774A.1, was cultured as a monolayer in DMEM supplemented with 10% FBS, 100 units/ml penicillin/streptomycin, and 2 mM L-glutamine (GIBCO). Another macrophage cell line, RAW 264.7 stably expressing HA tagged-TLR9, was cultured in RPMI-1640 (GIBCO) with the same supplements. Both cell lines were incubated with 0.8 μm blue-dyed latex beads (Sigma) for 1 h with a multiplicity of infection (MOI) at 50:1, washed with PBS, and incubated in medium for 1 h. Each cell internalized 5–10 microbeads. After cell lysis using a Dounce homogenizer, the bead-containing phagosomes were isolated on a sucrose gradient described by Desjardins *et al*. (14). The phagosomal band on top of the sucrose gradient (10%), as well as 3 additional bands (fractions 1, 2, and 3) at the interfaces beneath the gradient (25%, 35%, and 60%) were collected [Fig. S1]. Fractions 1, 2, and 3 were subjected to TCA-mediated protein precipitation followed by an acetone wash, and then were centrifuged to acquire protein pellets. The pellets were dissolved in 4% SDS in 50-mM Tris-HCl buffer (pH 8.0) by vortexing them on an agitator (Eppendorf) at 1,400 rpm for 1 h at 20 °C. Protein solutions obtained from the supernatant after centrifugation (14,000 rpm $\times$ 15 min at 4 °C) were used for immunoblots.

The phagosomal fraction was resuspended in a PBS containing a protease inhibitor mixture (Calbiochem), and washed by ultracentrifugation. The pelleted phagosomes were either resuspended in 4% SDS buffer for total protein extraction, as described above, or subjected to further fractionation for enriching membrane-bound proteins by using the following protocol. Washed phagosomes were re-suspended in 0.2 M Na_2CO_3 (pH 11.0), and the organelle membranes were disrupted by 5–7 passages of the suspension through a 25-G syringe needle. The resulting sample was left on ice for 30 min before the membrane fraction was pelleted by centrifugation for 45 min at 200,600 $\times$ *g* at 4 °C. Luminal

soluble proteins from the supernatant were precipitated and re-dissolved similarly as the proteins in fraction B1–B3. The phagosomal membrane pellet was resuspended in 4% SDS buffer for protein extraction as described above. Protein concentration was determined by the BCA assay (Piece).

SDS/PAGE and In-gel Digestion. Membrane-extracted proteins (35 μg) were separated by SDS/PAGE (4–12%, Bio-Rad). The entire Coomassie-stained gel was cut into 23 consecutive bands, and the gel slices were subjected to in-gel digestion (38). Biological duplicates were prepared for analysis by using 2 types of mass spectrometers.

Protein Identification by LC-MS/MS and Data Analysis. The in-gel digestion of each band was analyzed on either a Q-TOF (QSTAR®, Applied Biosystems) or a linear ion trap (LTQ, Thermo Inc.). LC-MS/MS data were searched against data in the International Protein Index (IPI) mouse database (v3.24 $>$ 50,000 entries) by using Mascot (v2.1, Matrix Science). Analytical details of LC-MS/MS and Mascot search parameters are provided in *SI Materials and Methods*. Each protein ID was assigned a major cellular function by GO-Getter (http://bmf2.colorado.edu/go-getter/help.psp) based on Genome Ontology (GO) terms. The number of transmembrane helices in each protein was predicted by using the TMHMM program. (http://www.cbs.dtu.dk/services/TMHMM/).

Autophagy Induction by Starvation and Drug Treatment for Biochemical Study. J774 cells were washed with PBS and incubated in the starvation medium, Earle's balanced salts solution (EBSS, Invitrogen), for 2 h at 37 °C (31). Vinblastine (Sigma) was added to EBSS at 50 μM to accumulate autophagosomes (39). Alternatively, protease inhibitors, E64 (10 μM) and pepstatin A (2 μM), were added to EBSS to prevent degradation of autophagosomal components. 3-Methyladenine (Sigma) was added to EBSS at 10 mM for autophagy inhibition (32). Cells were harvested after 2 h starvation, either in the presence, or in the absence, of a particular drug.

Subcellular fractionation from autophagy-induced J774 cells was also performed to acquire phagosomes in the same manner described earlier.

Autophagy Induction for Live Cell Imaging. A macrophage cell line stably expressing GFP-LC3 (kindly provided by Patrick Fitzgerald, St Jude's Children's Research Hospital, Memphis, TN) was used here. Cells were seeded on Nunc Lab-Tek Chambered Coverglass microscopy slides (Fisher) 20 h before the imaging experiment. After incubation with latex beads (3.0 μm microspheres, Polysciences) for 1 h and extensive washes, the cells were treated with either chloroquine (50 μM) or 3-MA (10 mM) for 2 h. During the final 15 min, LysoTracker Red DND-99 (Molecular Probes) was added at 100 nM. Cells were washed with PBS and treated with 15 μg/ml Hoechst 33342 (Molecular Probes) in PBS for 2 min. Finally, the live cells were rinsed and imaged by using a Zeiss 200-M epi-fluorescent microscope. All images were deconvolved by using the nearest neighbor deconvolution algorithm from the instrument software Slidebook 4.2 (Intelligent Imaging Innovations).

Immunoblots. The total lysate from starved and drug-treated cells was obtained by sonicating cells in a 2% SDS, 50 mM Tris-HCl buffer (pH 8.0) with protease inhibitor mixture (Calbiochem). After centrifugation, the supernatant was collected, and the protein was quantified by the BCA assay. Protein extracts were separated by SDS/PAGE and analyzed by western blots with relevant antibodies.

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